THE PRODUCTION OF NORMAL EMBRYOS BY ARTIFICIAL PARTHENOGENESIS IN THE ECHIUROID, *URECHIS*

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Introduction

The eggs of annelids and mollusks have been the subject of numerous parthenogenesis experiments, but only a few instances have been reported in which normal development was said to have been obtained. Lefevre (1907) described and figured parthenogenetically produced embryos of *Thalassema mellita* which resembled the normal, but he states (p. 116), "I have never observed a single instance where the trochophores rose to the surface of the water." Just (1915) reports the production of "normal-looking swimming forms—trochophores scarcely to be distinguished from the normal"—from eggs of *Nereis* exposed to high temperature. Morris (1917) obtained embryos which she described as "fairly normal swimming larvæ" from eggs of *Cumingia* exposed to high temperature and hypertonic sea water. In other cases the embryos obtained were described merely as swimmers or even uncleaved swimmers, (Allyn, 1912; Loeb, 1908, 1912; Lillie, 1910; Kostanecki, 1908; Bullot, 1904; Scott, 1906; Treadwell, 1902; Fischer, 1903). It is evident from the above citations that normal embryos have rarely, if at all, been obtained. The question may then be raised as to whether the sperm performs some special function in the eggs of annelids and mollusks, such that in its absence normal development is less likely to be obtained.

In the eggs of some annelids and mollusks there is evidence that the entrance point of the sperm is instrumental in determining the plane of bilateral symmetry of the embryo (Just, 1912; Morgan and Tyler, 1930). Such an orienting point is lacking, of course, in eggs treated with the usual parthenogenetic agents. It is important to know, therefore, whether eggs, in which the entrance point of the sperm is a factor in the determination of bilateral symmetry, are capable of normal development after artificial activation.

For the eggs of *Urechis* it was found that the first cleavage plane coincided with the entrance point of the sperm in the great majority of cases. Since in spirally cleaving eggs the first cleavage plane bears

a definite relation to the plane of bilateral symmetry of the embryo, it may be safe to conclude that the sperm entrance point determines the orientation of the embryo in *Urechis*. The eggs of *Urechis* can be activated by means of certain artificial agents; such as, hypotonic sea water, hypertonic sea water and high temperature. Top-swimming embryos indistinguishable from those produced by normally fertilized eggs were obtained from eggs treated with hypotonic sea water. However, the normal embryos were produced in very small numbers, the various types of abnormal swimmers being far more abundant.

The eggs which are to cleave and develop can be distinguished and isolated from the others at a few minutes after treatment. The manner in which these eggs respond to the treatment presents a striking contradiction to the view (Just, 1922) that normal development results only when the initial response of the egg to the artificial agent is similar to its response to the sperm.

Material and Methods

Urechis caupo was found at Monterey Bay and described by Fisher and MacGinitie (1928). Professor MacGinitie later found the same species at Newport Bay and I am indebted to him for the information on the location of the animals and the method of obtaining the sexual products from them. This remarkable echiuroid is ripe practically the year round, and one pair of animals may supply eggs and sperm for a number of experiments. The ability to obtain eggs repeatedly from the same individual would seem to cut down the variability of different experiments. However, it was found that the eggs of a segmental tube, from which samples had previously been removed, decrease in size after a couple of weeks and their response to fertilization is not quite 100 per cent, as is the case when eggs are removed from an untouched tube.

The agents used to activate the eggs were diluted sea water, distilled water, concentrated sea water and high temperature, but since the first was mostly used in these experiments, it alone will be described. The dilutions of sea water were made up of distilled water and sea water taken at a definite height of tide. The eggs were washed twice and allowed to settle in a centrifuge tube. The supernatant sea water was then removed and the eggs together with 0.3 to 0.5 cc. of sea water transferred to 50 cc. of the diluted sea water. Eggs were then removed after various intervals of time to 10 cc. of sea water by means of a small bore pipette filled to 0.2 cc. Any dilution of sea water up to 80 per cent was found to be capable of activating the eggs. Controls of unfertilized eggs kept in covered dishes gave no

activation and controls of eggs inseminated at the time of the experiment gave 95 to 100 per cent fertilization and development.[1]

Normal Fertilization and Development

The development of the fertilized egg of *Urechis* is so similar to that of *Thalassema* (Torrey, 1902, 1903) that only a brief description need be given here for comparison with the parthenogenetic eggs. The "fertilization reaction" differs somewhat from that described for other marine ova, and since use was made of this reaction in the entrance point observations, it will be described below.

The unfertilized egg of *Urechis* (Fig. 1) has a rather unique shape. It has a large indentation which varies somewhat in size in different eggs. This shape is similar to that which a spherical rubber ball would assume if it were evacuated and filled with a volume of water about one-fourth less than $\frac{4}{3}\pi R^3$. The ratio of surface to volume is thus much greater than for a spherical egg. Eggs with two indentations (Fig. 2) may also be found, but if such eggs are made to round out in dilute sea water and returned to normal sea water only one indentation reappears.

The unfertilized egg contains a large germinal vesicle and a single nucleolus. The germinal vesicle is nearest the surface of the egg at the innermost point of the indentation. This point, as will be shown below, marks the pole of the egg, and the egg may be seen to be radially symmetrical about this axis (Fig. 3).

The sperm enters the egg at any point on the surface with respect to the indentation. Within three minutes after the attachment of the sperm, a clear conical process appears on the egg immediately below the point of attachment.[2] The cone enlarges within the next two minutes and the membrane immediately above it becomes thin (Fig. 4). At this time the indentation begins to round out and the fertilization membrane begins to separate from the surface of the egg. The sperm head then enters the cone (Fig. 5) and in about 90 seconds passes into the egg (Fig. 6), the tail remaining behind. During the next five minutes the cone continues to enlarge and becomes cylindrical rather than cone-shaped, with a rounded outer end (Fig. 7). It enlarges more rapidly than the perivitelline space, apparently stretching the membrane above it. The "cone" then begins to narrow considerably in width so that at about thirteen minutes after the entrance of the sperm it has the appearance of a single filament

[1] The insemination was usually done in another part of the room by Betty S. Tyler.

[2] This time schedule holds for 20° C., unless otherwise stated.

(Fig. 8). It finally disappears at about seventeen to twenty-two minutes after the penetration of the sperm. The egg thus supplies a marker of the entrance point which can be seen as late as twenty minutes after insemination. Entrance point observations are therefore quite easy to make.

The first polar body is extruded 34 minutes after fertilization and the second at 44 minutes. Cleavage begins at 74 minutes after fertilization.[3] The first two divisions are equal. At the third cleavage the first quartet of micromeres arises dexiotropically. They are very slightly smaller than the macromeres. In the formation of the embryo, no essential differences were found from Torrey's description for *Thalassema*.

The Indentation and the Pole of the Egg

It is generally conceded that the polarity of an egg is established in the ovary, the polar axis being indicated by the eccentric position of the nucleus and radial arrangement of cytoplasmic materials about it. In the egg of *Urechis* the germinal vesicle is nearest the surface at the innermost point of the indentation (Fig. 1). If the indentation

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Plate 1

Photomicrographs of the living objects. The magnification is 330 diameters for all the figures with the exception of Fig. 1, for which the magnification is 300 diameters.

Fig. 1. Unfertilized egg in "side" view, showing indentation, large germinal vesicle and nucleolus.

Fig. 2. Unfertilized egg in "side" view showing two indentations.

Fig. 3. Unfertilized egg, antipolar view.

Fig. 4. Formation of fertilization "cone" at point of attachment of sperm, and rounding out of indentation.

Fig. 5. Showing sperm within cone, beginning of dissolution of germinal vesicle and elevation of membrane.

Fig. 6. Showing sperm head half-way in egg, enlargement of "cone," and further elevation of membrane.

Fig. 7. Showing persistence and enlargement of the "cone" five minutes after entrance of sperm head into egg.

Fig. 8. Showing the shrinkage of the "cone" into a filament-like process, thirteen minutes after entrance of sperm, and disappearance of germinal vesicle except for nucleolus.

Fig. 9. Showing eccentric position of germinal vesicle in an egg in which the indentation was made to disappear by allowing it to swell in 50 per cent sea water.

Fig. 10. Showing migration of remains of germinal vesicle to pole as marked by indentation; entrance point of sperm shown by filament-like cone near antipole of egg fifteen minutes after entrance of sperm.

Fig. 11. Photographed in the same position as in Fig. 10; shows formation of first polar body at center of formerly indented area 32 minutes after fertilization.

Fig. 12. Photographed in the same position as in Fig. 11; shows formation of second polar body next to first; 44 minutes after fertilization.

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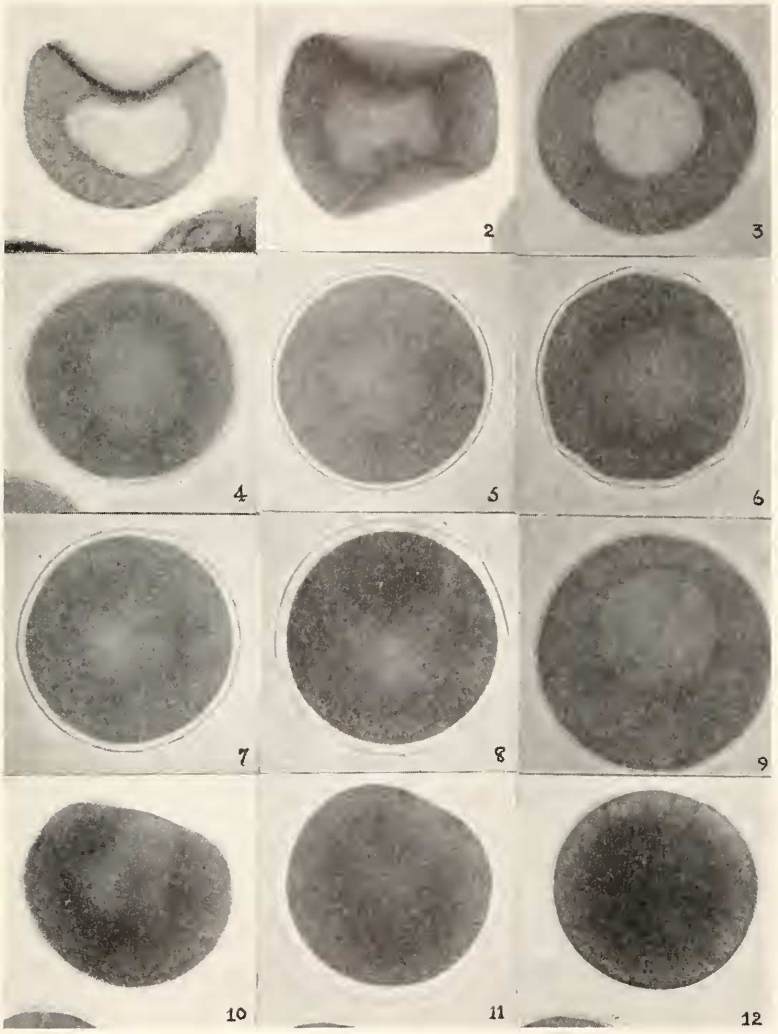
[3] At room temperature, which is generally about 23.5° C., the time for first cleavage is 65 minutes.

PLATE I.



is caused to round out by placing the egg in 50 per cent sea water, the germinal vesicle is seen to occupy an eccentric position in the egg (Fig. 9). The rounding out can be followed under the microscope, and the results of such observations show invariably that the eccentricity is the same as that of the original indented egg. This would indicate that the indentation marks the pole of the egg.

In order to obtain a more definite check on this relation, the point of extrusion of the polar bodies was determined on eggs in which the location of the indentation had been previously noted. The method used was identical with that described below for the entrance-point observations. The eggs selected for observations were those in which the indentation was on the side and thus distinctly visible. They were followed almost continuously throughout the time of rounding up and membrane elevation. Errors due to rotation of the egg were also partly guarded against by the presence of extra sperm which served as markers on the surface of the membrane. As the egg rounds up, the remains of the germinal vesicle are seen to migrate towards the surface of the egg in the region of the former indentation (Fig. 10). The first polar body later appears at about the center of that area on the surface (Fig. 11), and the second polar body is later extruded next to it (Fig. 12). The results of the observations given in Table I show four exceptions. These presumably represent cases in which the egg rotated within the membrane. However, there does not seem to be any general tendency for such rotation, since the polar bodies may appear in any position on the surface of the egg and remain in that position.

TABLE I

Relation between Indentation and Point of Extrusion of Polar Bodies

Divergence	No. of Eggs
0°– 10°	24
10°– 90°	2
90°–180°	2

RELATION OF THE ENTRANCE POINT OF THE SPERM TO THE PLANE OF BILATERAL SYMMETRY

The method used for making entrance point observations and the precautions to be taken have been described by Morgan and Tyler (1930). As has been mentioned above, the point of entrance of the sperm is very easily located in *Urechis* even as late as twenty minutes after fertilization. The first polar body appears ten minutes after the disappearance of the "cone" and serves as a marker on the surface of the egg. This diminishes the chances of unobserved rotation of the egg occurring.

The eggs were placed within a square of vaseline on a slide and the drop sealed over completely with a coverslip to prevent evaporation. This transfer was generally done at about ten minutes after fertilization. The first drawings were made five to ten minutes later, at which time the fertilization membrane has lifted off to about its full extent and the egg itself has become spherical. The position of the polar bodies was then noted as they appeared. At the time of cleavage the egg was watched almost continuously, since there is a tendency for eggs that are elongating vertically to roll over.

A number of the observations were made by Mr. Jackson Gregory, Jr., one of the students working at the Kerckhoff Marine Laboratory, and are listed separately in Table II as Series 1. The results of the observations given in this table show a coincidence of 71 per cent between the entrance point of the sperm and the first cleavage plane.

TABLE II

Relation between Entrance Point and First Cleavage Plane

Divergence	No. of Eggs		
	Series 1	Series 2	Total
0°–10°	61	66	127
10°–45°	13	24	37
45°–90°	8	6	14

This value is obviously very much higher than would be expected on a pure chance basis and clearly indicates that the position of the first cleavage plane is determined by the point of entrance of the sperm. The exceptions may be due to the inability of completely removing all sources of error or to the presence of abnormal eggs. The most convincing cases are those in which the entrance point and the polar axis lie in a horizontal plane on the slide. In order for coincidence to be obtained in such cases the egg must elongate vertically and the cleavage plane must cut through horizontally. Twenty-six eggs of that type were followed and in every case the first cleavage plane passed through the entrance point, although seven of them rotated during the division.

In eggs with spiral cleavage the plane of bilateral symmetry is given by the position of the 4*d* cell, which also marks the dorsal side of the embryo. The position of this cell is determined by the plane of first cleavage. In *Urechis* it can be located after its bilateral division

into M_1 and M_2, and its position is such as to cause the plane of bilateral symmetry to make an angle of about 60 degrees with the first cleavage plane.

Parthenogenesis Experiments

Activation by Dilute Sea Water

The eggs of *Urechis* may be activated by dilutions of sea water ranging from 80 per cent to distilled water. Table III gives the time range for the different solutions over which activation may be obtained. It is taken from a set of experiments run at temperatures between 20 and 22.5° C. The third column gives the time of exposure at which activation begins, taken arbitrarily as 5 per cent activation. These values are obtained graphically by connecting the two nearest points above and below 5 per cent with a straight line. The fourth column gives the time of exposure necessary to obtain 100 per cent activation. With longer exposures the percentage of activation drops again to zero for certain concentrations of dilute sea water. Column five gives the exposure at which the activation drops to 5 per cent, calculated in the same manner as for column three. Solutions below 45 per cent sea water cause cytolysis of the egg, the time for cytolysis varying inversely with the amount of dilution.

Table III

Range of Exposure Time in which Activation May be Obtained

Dilution of Sea Water	Temperature	Calculated Time for 5% Activation	Time for 100% Act.	Calculated Time for 5% Activation
per cent	° C.	*seconds*	*seconds*	*minutes*
0	21.8	1	12	
20	22.0	3	20	
30	22.1	10	30	
40	22.0	11	50	
45	20.1	21	60	12
50	22.5	15	60	10
55	20.1	43	100	10
60	21.5	35	105	15
65	22.3	48	150	16
70	21.2	64	180	19
75	20.8	87	210	9
80	22.5	74	210	

It is readily seen from Table III that the time for activation decreases with increased dilution of the sea water. This is consonant with an interpretation of activation based on the volume of water taken in, since water enters more rapidly the greater the dilution of sea water in which the egg is placed.

Two other points may be mentioned here which, together with their interpretation, will be reported in detail in a subsequent paper. The increase in activation occurs much more rapidly than the decrease, giving a skew activation-time curve. The percentage of the activated eggs which cleave varies with the time of exposure; but its variation is such that as the percentage of activation increases the percentage of cleavage decreases. Thus, when 100 per cent activation is obtained no cleavage occurs, while to either side of that exposure time the percentage of cleavage increases.

Production of Normal Embryos

Many of the cleaved eggs develop into swimming embryos, and a few into top swimmers, some of which are indistinguishable from those produced by normally developing fertilized eggs. Some of the experiments in which normal embryos were obtained are listed in Table IV.

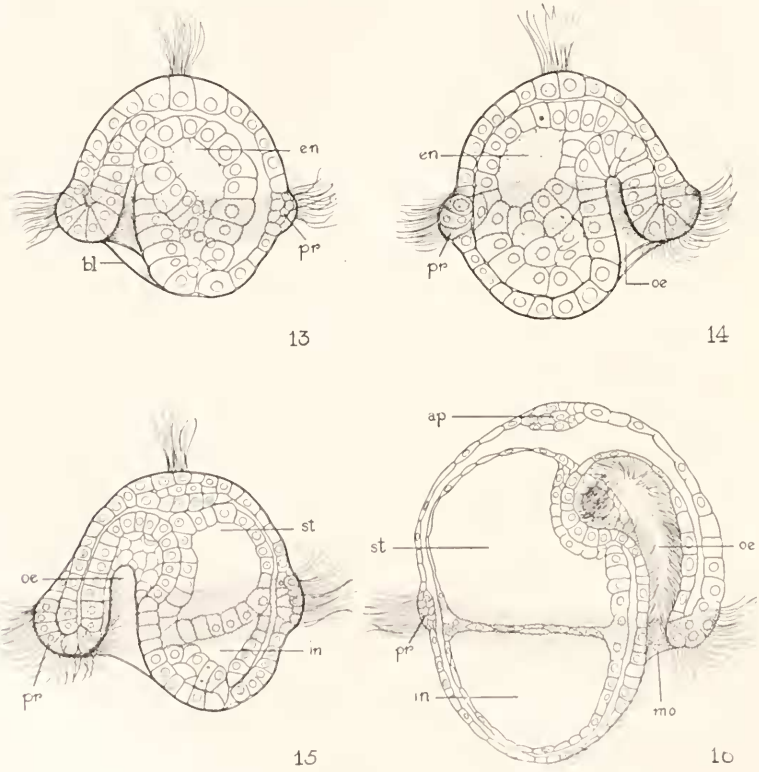
Table IV

Dilution of Sea Water	Exposure		Top Swimming Embryos	
			Normal	Abnormal
per cent	*minutes*	*seconds*		
	0	20	3	25
	1	10	1	14
30	1	0	3	29
	1	40	2	15
	2	20	5	41
	0	20	2	11
40	0	40	1	28
	1	0	1	14
	2	0	2	25
45	0	30	3	32
	1	15	1	15
	1	30	2	8
50	3	0	3	12
	6	0	3	17
60	4	0	3	26
	8	0	5	5
65	1	45	2	12
	4	0	1	5

As is shown by the table, the normal embryos are not produced by any particular dilution of sea water or any definite time of exposure. The percentage of normal development obtained is very small, most of the normal embryos listed occurring in dishes of five hundred to a thousand cleaved eggs.

In Figs. 13–16 some normal parthenogenetically produced embryos of various ages are presented. The embryos of fertilized eggs are not figured, inasmuch as they are identical with the parthenogenetic ones. The early gastrulation stages of the parthenogenetic embryos were not obtained, since at that time the normal embryos are not readily distinguishable from certain abnormal types to be described later.

The young trochophore of sixteen hours (Fig. 13) shows a large



Figs. 13–16.

ap, apical plate; *bl*, anterior portion of original blastopore; *en*, enteron; *in*, intestine; *mo*, mouth; *oe*, œsophagus; *pr*, prototroch; *st*, stomach. Normal embryos from artificially activated eggs; drawn from total mounts of preserved specimens.

Fig. 13. Sixteen-hour trochophore.

Fig. 14. Twenty-hour trochophore.

Fig. 15. Twenty-four-hour trochophore.

Fig. 16. Forty-eight-hour trochophore.

enteric mass in which a small cavity has appeared. The prototroch is well developed and the beginning of the œsophageal invagination is present below the prototroch on the ventral side of the embryo. In the twenty-hour trochophore (Fig. 14) the invagination has progressed towards the anterior end of the embryo and the post-trochal region has enlarged. The enteric cavity soon becomes divided into stomach and intestine (Fig. 15), and the œsophagus reaches the anterior end of the embryo. The whole embryo enlarges considerably during the next 24 hours and becomes a relatively thin-walled structure (Fig. 16). The anterior end of the œsophagus is turned to the right side of the stomach where it acquires a ciliated opening into the latter.

Bilateral symmetry is evident in the sixteen-hour embryo. The plane of bilateral symmetry is, as pointed out above, determined very much earlier, and for fertilized eggs, at the time of entrance of the sperm. The parthenogenetically activated eggs can evidently establish a plane of bilateral symmetry without the aid of a localized external agent such as the sperm entrance point. However, this is done in relatively very few instances, while in the great majority of cases, even with apparently normal cleavage, abnormal embryos of various types are obtained. Of particular interest in this connection is the occurrence of embryos that are more or less radially symmetrical.

Radially Symmetrical Embryos

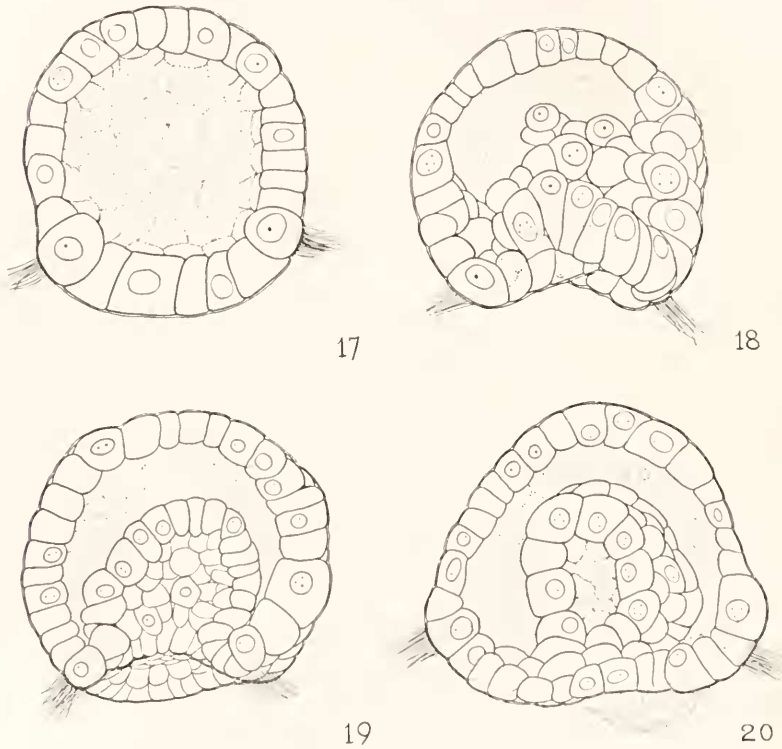
Among the top swimming and bottom swimming embryos produced from the artificially activated eggs, there occur a number of forms which may be grouped in a radially symmetrical class. They retain their radial form until their death, which occurs long after the attainment of bilateral symmetry in the normal embryos.

Certain of these embryos have the form of a hollow blastula (Fig. 17), resembling the blastula of the sea-urchin. The prototroch is well developed and large entoblast cells are present below it, but no gastrulation occurs at any time in its life history. Another type (Fig. 18) shows the beginning of gastrulation, which in some instances progresses to the formation of a large enteric mass (Fig. 19). In certain cases the enteric cavity may be formed (Fig. 20). In none of these types is there any outward evidence of bilateral symmetry.[4] The blastopore does not undergo its antero-ventral migration, and the œsophagus is not formed.

It is quite probable, although the cell-lineage has not been worked out, that these forms arise from eggs in which the normal bilateral

[4] Other forms have been observed which might be classed as transition types, but their abnormal form makes them quite difficult to analyze.

cleavages do not take place. Torrey (1903) has described eggs of *Thalassema* that have more or less completely reverted to the radial type. He states, "There is, also, strong reason for believing that this type never develops into an adult; for I found a few gastrula stages in which no X group, no M cells, no larval mesenchyme cells from the third quartet and no large anterior œsophagoblast ($2b_{2.2+}$) could be distinguished." It may be fairly safe to assume then that the cell lineage of the radial embryos described above is similar to that described by Torrey, and that the failure to develop into a bilateral



FIGS. 17–20. Radially symmetrical embryos from artificially activated eggs; drawn from total mounts of preserved specimens.

FIG. 17. Hollow blastula of 24 hours.

FIG. 18. Beginning gastrula of 22 hours.

FIG. 19. Gastrula of 28 hours, showing large enteric mass.

FIG. 20. Gastrula of 28 hours, showing enteric cavity.

trochophore results from the failure of certain bilateral cleavages to occur, so that no X group, M cells, etc. are formed.

Approximately one-quarter of the top swimming embryos and about 5 per cent of the bottom swimmers were radially symmetrical

or approached the radial type. No radial embryos were observed among the trochophores of the normally fertilized eggs. Their occurrence as a result of artificial activation may be taken as an expression of the inability of the parthenogenetically activated egg to completely negotiate its problem of bilateral symmetry. The low percentage of normal development obtained is then accountable by the assumption that it is a matter of chance whether the response of the egg is similar to that resulting from the entrance of the sperm or whether it responds in an "abnormal" fashion to the artificial treatment. Before this could be put in more concrete terms, we would have to know much more about the response of the egg to the sperm in cases where the entrance point determines the median plane of the embryo.

Maturation and Cleavage of the Artificially Activated Eggs

It has been previously noted that 100 per cent activation may be obtained with certain times of exposure to different dilutions of sea water, but that the percentage of cleavage decreased with increased activation so that at the optimum point practically no cleavage is obtained. The eggs at the optimum point were all found to extrude two polar bodies,[5] whereas on either side of that exposure time increasing numbers of eggs with one and with no polar bodies were found. Eggs were therefore isolated according to the number of polar bodies formed in order to determine which type cleaved and developed.

Table V

Relation between Cleavage and Number of Polar Bodies Formed

Type of Egg Isolated	Number of Eggs Cleaved	Number of Eggs Uncleaved
No polar bodies	876	156
One polar body	0	83
Two polar bodies	0	518

The results obtained by isolating the various types of eggs are given in Table V. The eggs were isolated at 45 to 75 minutes after treatment and cleavage recorded about three hours later. A binocular microscope magnifying 125 diameters was used in making the isolations and the eggs were rolled around to make sure that the presence of polar bodies was not overlooked. None of the eggs with one or with

[5] The first polar body may divide in about half of the eggs, as is the case in the fertilized eggs, so that when eggs with two polar bodies are referred to in the text it is meant to include also those with three polar bodies.

two polar bodies were found to divide, whereas 85 per cent of the eggs with no polar bodies divided. This result is in accord with that obtained by Morris (1917) on the eggs of *Cumingia*. She found that a high percentage of maturation resulted in a low percentage of cleavage stages and larvae, and from 1215 eggs with no polar bodies obtained five larvae, whereas from 313 eggs with one or two polar bodies one embryo developed that was evidently uncleaved.

In *Urechis* the eggs with one or with two polar bodies can be readily distinguished from those with none. The former are decidedly more "normal" in appearance and in their reaction to the treatment than are the latter. It is quite surprising then that only the abnormal-looking eggs with no polar bodies develop, whereas the others do not even cleave. These types of eggs will now be described in some detail.

When the eggs are placed in the dilute sea water, the indentation rounds out and the whole egg swells due to the intake of water (Fig. 9).[6] Upon return to normal sea water the egg shrinks and the indentation reappears. The egg thus returns to its original shape. This is true for all the eggs so treated regardless of their subsequent history.

PLATE II

Photomicrographs of living objects. The magnification is 330 diameters.

FIG. 21. Artificially activated egg five minutes after return to normal sea water, showing beginning of membrane elevation and rounding of indentation.

FIG. 22. Artificially activated egg ten minutes after return to normal sea water, showing beginning of dissolution of germinal vesicle.

FIG. 23. Artificially activated egg fifteen minutes after return to normal sea water. Compare with Fig. 10 of fertilized egg.

FIG. 24. Artificially activated egg 34 minutes after return to normal sea water, showing first polar body.

FIG. 25. Artificially activated egg 47 minutes after return to normal sea water, showing second polar body.

FIG. 26. Artificially activated egg 50 minutes after return to normal sea water, showing single giant polar body.

FIG. 27. Artificially activated egg 45 minutes after return to normal sea water, showing presence of indentation, disappearance of germinal vesicle, and absence of membrane and of polar bodies.

FIG. 28. Antipolar view of an egg of the type shown in Fig. 27, showing small nucleus.

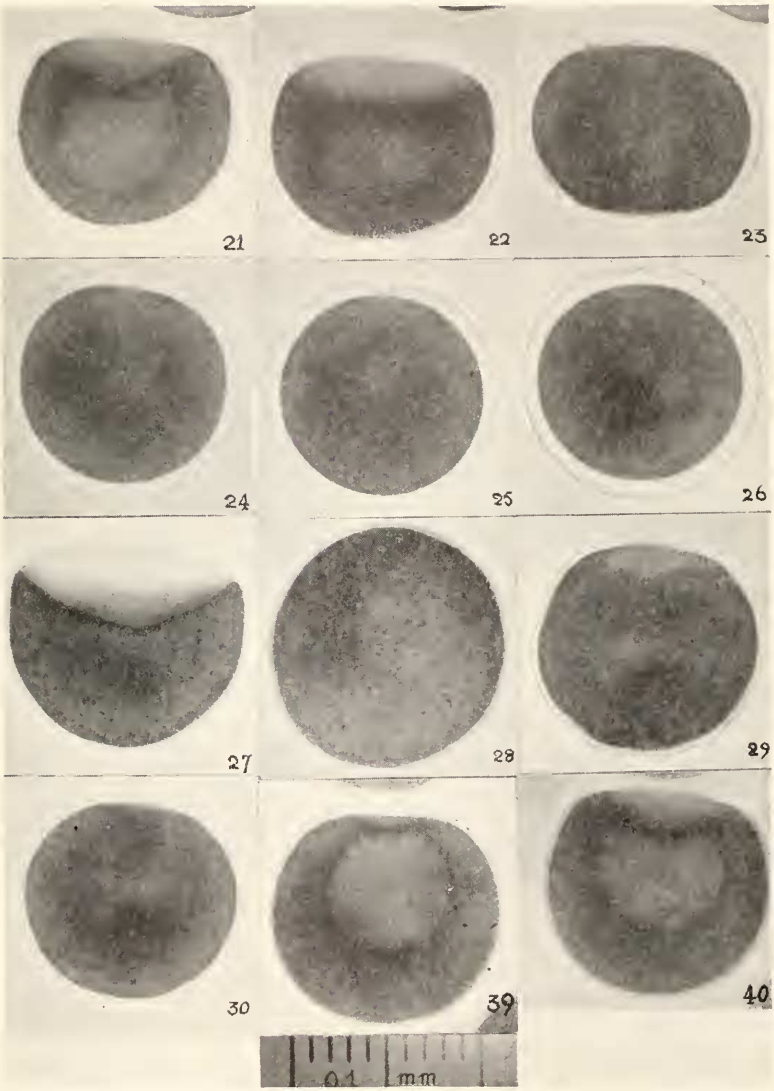
FIG. 29. Same egg as in Fig. 27, 40 minutes later, showing rounding up of egg, elevation of membrane, and formation of cleavage spindle (indicated by clear area) at right angles to polar axis.

FIG. 30. Same egg as in Fig. 27, 5 minutes later, completely rounded up.

FIGS. 39 AND 40. Show reappearance of indentation in its original position when egg is returned to normal sea water after treatment with dilute sea water.

[6] In dilutions of sea water above 65 per cent the indentation does not completely round out.

PLATE II.



It has been noted above that with certain lengths of exposures to dilute sea water, the time of treatment depending upon the concentration employed, 100 per cent of the eggs become activated. Such eggs upon return to normal sea water behave very much as though they had been normally fertilized except that no entrance cone is formed. The germinal vesicle breaks down, the indentation rounds out, and the membrane is elevated very much in the same manner as in the fertilized eggs (Figs. 21, 22, and 23). The first and second polar bodies (Figs. 24, 25) appear at the same time as they do in the fertilized eggs, if allowance is made for the time of return to normal shape in sea water.

This last point is illustrated in Table VI. The figures given in

TABLE VI

Time of Polar Body Extrusion in the Artificially Activated Eggs

Dilution of Sea Water	Temperature	Length of Exposure	Time for First Polar Body		Time for Second Polar Body	
			Total	Calculated	Total	Calculated
per cent	° C.	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
30	20.0	$\frac{1}{3}$	35	$34\frac{1}{2}$	46	$45\frac{1}{2}$
		1	35	$33\frac{1}{2}$	47	$45\frac{1}{2}$
		$2\frac{1}{2}$	36	$32\frac{1}{4}$	50	$46\frac{1}{4}$
		$4\frac{1}{2}$	41	$34\frac{1}{4}$	55	$48\frac{1}{4}$
Fert. eggs				34		44
45	23.5	$\frac{1}{3}$	$30\frac{1}{2}$	30	41	$40\frac{1}{2}$
		1	31	$29\frac{1}{2}$	42	$40\frac{1}{2}$
		$2\frac{1}{2}$	33	$29\frac{1}{4}$	43	$39\frac{3}{4}$
		$4\frac{1}{2}$	$36\frac{1}{2}$	$29\frac{3}{4}$	48	$41\frac{1}{4}$
Fert. eggs				30		40
50	19.5	1	36	$34\frac{1}{2}$	48	$46\frac{1}{2}$
		$1\frac{2}{3}$	$37\frac{1}{2}$	35	$49\frac{1}{2}$	47
		$2\frac{1}{2}$	38	$34\frac{1}{4}$	51	$47\frac{1}{4}$
		$4\frac{1}{2}$	$39\frac{1}{4}$	$32\frac{1}{2}$	$52\frac{1}{2}$	$45\frac{3}{4}$
Fert. eggs				$34\frac{1}{2}$		46

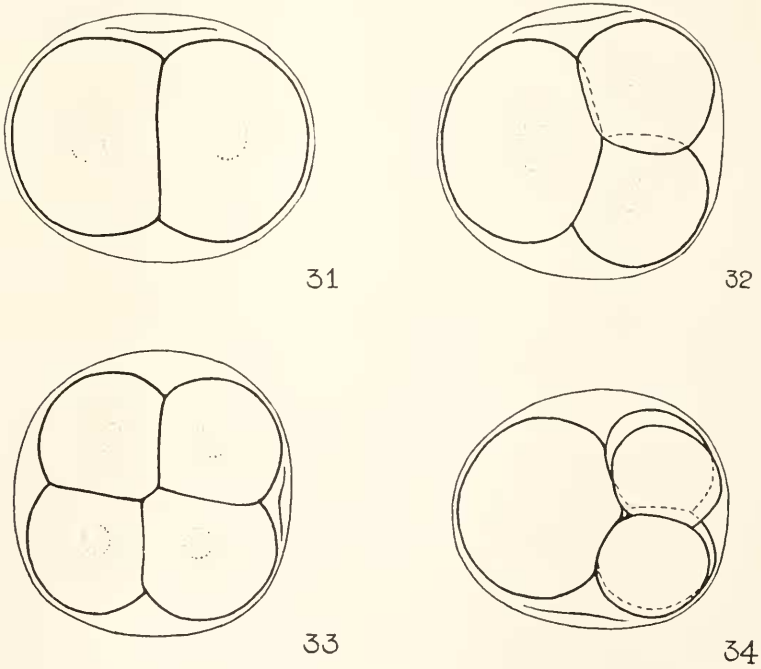
* The total time is taken from the beginning of the treatment.

† The calculated time is that obtained by allowing for the swelling and shrinking of the egg.

columns four and six of the table are for the time of appearance of the polar bodies from the beginning of the treatment. If allowance is made for the time of exposure, the time for appearance of the polar bodies still increases with the length of exposure. We must therefore subtract an amount equal to the time for return to original shape. A rough determination of the time of shrinkage shows it to be about

half the swelling time. Allowing for that, the time of appearance of the polar bodies is comparable with the time of polar body extrusion in the fertilized eggs. This holds for all eggs that form two polar bodies, whether or not they have received the exposure resulting in 100 per cent activation. It is evident from these results that development does not start in the dilute sea water but after its return to normal sea water. This is in accord with the fact that the egg first assumes its original form on return to normal sea water and then proceeds to develop. It is also consistent with the fact (to be reported in a subsequent paper) that eggs which have been "over exposed" to dilute sea water do not become activated although they are still capable of fertilization.

The eggs which extrude only one polar body occur in much fewer numbers. The maximum amount observed was about one per cent of the activated eggs. When only one polar body is extruded it is



FIGS. 31–34. Early cleavages of artificially activated eggs, drawn from photographs of living objects.

FIG. 31. Two-cell stage of an artificially activated egg.

FIG. 32. Three-cell stage of an artificially activated egg.

FIG. 33. Four-cell stage of an artificially activated egg, showing polar cross-furrow.

FIG. 34. Five-cell stage produced by the egg shown in Fig. 32.

generally quite large, being approximately equal in size to both polar bodies of the normal egg (Fig. 26). The average time of extrusion of the polar body was 45 minutes, which is the time of second maturation division in the normal egg.

The eggs that produce no polar bodies respond to the treatment in such a way that they were first classified as "poorly activated." The germinal vesicle disappears much more slowly than in the others. The rounding out of the indentation and elevation of the membrane may not occur for one to two hours. Figure 27 shows such an egg 45 minutes after treatment. Although the germinal vesicle has disappeared, no membrane is present and the indentation remains.

A small nucleus is later formed which persists until the time of rounding up of the egg (Fig. 28). The time at which the egg rounds up varies from 40 minutes to over two hours. As the indentation disappears an irregular membrane is lifted off from the surface of the egg (Figs. 29 and 30) and a spindle develops at right angles to the egg axis. Within 20 minutes after the rounding up, the egg elongates and divides (Fig. 31).

The first division may be equal or unequal. Of the 876 divided eggs listed in Table V, 485 had formed an equal or nearly equal two-cell stage. Four of the eggs had divided at once into three cells and the rest had formed an unequal two-cell stage. The next division of both the equally cleaved and unequally cleaved eggs may be into three cells (Fig. 32) or into four cells (Fig. 33). The time of division is quite irregular. Table VII gives the time schedule of division for

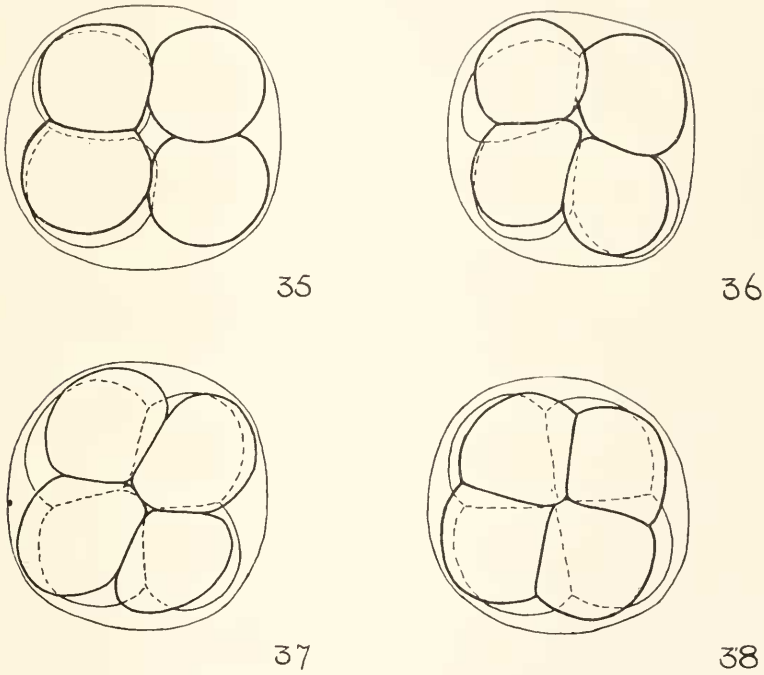
TABLE VII

Number of Eggs in Different Cleavage Stages at Various Times after Treatment—from Isolation of Uncleaved Eggs

Time after Treatment		1-cell	2-cell	3-cell	4-cell
hours	*minutes*				
1	10	300	0	0	0
1	20	298	2	0	0
1	35	270	30	0	0
1	45	267	31	2	0
2	0	202	96	2	0
2	30	112	160	24	4
3	0	30	201	41	28

300 eggs without polar bodies that were isolated before cleavage began. The table shows that the behaviour of the eggs with respect to time of division is far from uniform; the second division in some eggs may occur even before the first division in others.

The eggs which divide into three cells at the second division generally divide into five at the next (Fig. 34), the original undivided cell still remaining undivided. Those which divide into four cells at the second division may divide into six, seven, or eight cells at the third cleavage (Figs. 35, 36, 37, 38). The third cleavage is dexiotropic as in the normal egg. The four-cell stage also shows evidence of spiral cleavage in the presence of the cross furrow, which results from the *A* and *C* blastomeres being higher than the *B* and *D*. The time of third cleavage is also quite variable. In order to determine whether



FIGS. 35–38. Early cleavages of artificially activated eggs, drawn from photographs of living objects.

FIG. 35. Six-cell stage produced by the type of egg shown in Fig. 33.

FIG. 36. Seven-cell stage produced by the type of egg shown in Fig. 33.

FIG. 37. Eight-cell stage produced by the type of egg shown in Fig. 33; showing dexiotropic cleavage.

FIG. 38. Later eight-cell stage showing dexiotropic cleavage.

a more uniform result might be obtained, equally cleaved eggs were isolated according to whether they divided early or late. The results of two such experiments are given in Tables VIII and IX. No significant differences in types of cleavage obtained are evident, and no greater uniformity in time of division results, when the divided

TABLE VIII

Number of Eggs in Different Cleavage Stages at Various Times after Treatment—from Equally Cleaved Eggs Isolated Early

Time after Treatment	2-cell	3-cell	4-cell	5-cell	6-cell	7-cell	8-cell
1 hour 50 min. to 2 hrs. 15 min.....	190	0	0	0	0	0	0
2 hours 30 min.....	168	22	0	0	0	0	0
2 " 47 "	143	47	0	0	0	0	0
2 " 53 "	129	47	14	0	0	0	0
3 " 0 "	106	60	24	0	0	0	0
3 " 5 "	106	50	24	10	0	0	0
3 " 10 "	92	59	26	10	0	3	0
3 " 30 "	56	48	39	22	6	11	8
4 " 0 "	21	36	31	38	22	17	25

eggs are isolated early or late. The normal-looking eggs in the eight-cell stage were isolated from the early dividing and late dividing series. The embryos produced were examined about 32 hours later.

TABLE IX

Number of Eggs in Different Cleavage Stages at Various Times after Treatment—from Equally Cleaved Eggs Isolated Late

Time after Treatment	2-cell	3-cell	4-cell	5-cell	6-cell	7-cell	8-cell
2 hours 20 min. to 2 hrs. 45 min.....	207	0	0	0	0	0	0
2 hours 52 min.....	184	0	23	0	0	0	0
3 " 0 "	159	25	23	0	0	0	0
3 " 15 "	72	86	49	0	0	0	0
3 " 35 "	64	86	57	0	0	0	0
3 " 50 "	53	74	51	15	4	4	6
4 " 15 "	24	58	62	29	11	10	13

The results are listed in Table X. It may be seen that the retarded eggs behave as well with respect to the type of embryos produced as the early dividing ones. The table also shows that only very few of the eggs that form a normal-looking eight-cell stage develop into

TABLE X

Embryos Produced by Normal-Looking Eggs Isolated in the Eight-Cell Stage

Type of Egg	Number Isolated	Normal Embryos	Top Swimmers	Bottom Swimmers
Early dividing	57	1	8	30
Late dividing	78	1	5	45

normal embryos. It might be claimed that this is due to irregularities in the division of the chromosomes, but such irregularities would probably manifest themselves in abnormal divisions before that time. This point will, however, be checked when a study of the chromosomes of the artificially activated eggs is made.

POLARITY OF THE ARTIFICIALLY ACTIVATED EGGS

The abnormal development might also be interpreted on the basis of a disturbance in the polarity of the egg. However, assuming that the indentation marked the pole of the unfertilized egg, it was found that the polarity of the artificially activated eggs remained unchanged. These observations were made by means of the vaseline-slide method. When the eggs are allowed to swell in the dilute sea water, the indentation rounds out, and the germinal vesicle is nearest the surface at the formerly indented region (Fig. 9). Upon return to normal sea water the indentation reappears. The reappearance of the indentation can be followed quite easily (Figs. 39 and 40) with respect to the germinal vesicle and proves to occupy the same position as in the original egg. In the eggs which form polar bodies as a result of the treatment, the indentation soon rounds out and the membrane is elevated (Figs. 21, 22, and 23). The contents of the dissolved germinal vesicle moves towards the pole (as determined by the indentation) and the first polar body later appears at that point (Fig. 24). Fourteen eggs were followed in the manner described and all gave the same results. In the eggs which do not form polar bodies, the indentation persists for some time. Eighteen eggs of that type were followed to the first division and in every case the first cleavage plane passed through the polar axis (as determined by the indentation). However, if the original polarity is retained, the second cleavage plane

must also coincide with the polar axis. This was found to be true for all of six eggs followed to the second division. It appears then that the abnormal development cannot be attributed to altered polarity of the egg.

Discussion

In the eggs of *Urechis* the sperm appears to be important for the determination of the plane of bilateral symmetry. The small percentage of normal embryos obtained from the artificially activated eggs may then be interpreted as due to the failure of the processes connected with the establishment of bilaterality to occur in all of the eggs, although they may take place "by chance" in some of the eggs. The production of radially symmetrical embryos from the treated eggs is consistent with such an interpretation. In other eggs with spiral cleavage (*Nereis*, *Cumingia*, and *Chætopterus*) there is evidence for the determination of the plane of bilateral symmetry by the entrance point of the sperm. In such eggs development closely simulating the normal has been shown to be quite difficult to obtain, although the amount of normal-looking cleavage obtained is generally quite considerable.

The view expressed here might presumably be tested by the use of an artificial agent the action of which on the egg is more nearly like that of the sperm. The needle of the puncture method may be taken to be such an agent. Brachet (1911) has studied the relation of the point of puncture to the plane of bilateral symmetry in the egg of the frog. In the frog's egg the gray crescent has been shown to form opposite the entrance point of the sperm, and the plane of bilateral symmetry is determined by the position of the crescent. Brachet found that in the punctured frog's egg the position of the crescent has no definite relation to the point of puncture. In those eggs that develop, the median plane forms in relation to the crescent. This would appear then to be evidence against the idea expressed above, and Brachet interpreted the result to mean that the egg itself has a sort of labile bilaterality. However, he points out that the action of the needle is not quite comparable to that of the sperm. He states (p. 357), "le stylet, si fin qu'il soit, est un instrument grossier, dont l'action est plus brutale et surtout beaucoup plus rapide; si habile que soit l'operateur, le fil de platine ou de verre a atteint le centre de l'œuf en une fraction de seconde. Aussi l'œuf reágit-il en masse, et presque simultanément dans toutes ses parties, tout comme quand il se sent pénétré en même temps par 8 ou 10 spermatozoïdes." He suggests using a very fine needle of one to five micra thickness, and allowing it to take ten to fifteen minutes to reach the center of the upper hemisphere of the egg.

The failure of the eggs that extrude two polar bodies to develop has been noted in one other case of artificial parthenogenesis in annelids and mollusks. Morris (1917) found in *Cumingia* that very few eggs with one polar body or with two polar bodies divide, whereas those with no polar bodies may develop into swimming embryos. This result was confirmed by Heilbrunn (1925). In other cases it has been reported that development may proceed whether one, two or no polar bodies are formed. This has been noted for *Mactra* (Kostanecki, 1904, 1911), *Thalassema* (Lefevre, 1907), *Asterias* (Delage, 1901, 1904; Garbowski, 1903), *Amphitrite* (Scott, 1906), and *Chætopterus* (Allyn, 1912; Lillie, 1906).

In *Urechis*, one of the most interesting features of the eggs which fail to extrude polar bodies is their slow and comparatively abnormal response to the treatment. Those that extrude polar bodies respond to treatment as well as though they had been fertilized. It is surprising then that only the former should develop, since it has been generally held that the degree of success attained by artificial agents depends on the extent to which the initial response of the egg to the treatment resembles its response to the sperm. Just (1922), for example, states, "the highest per cent and normality of cleavage and of plutei result when the membrane separation most closely simulates the separation of the vitelline membrane as a cortical response to insemination." In *Urechis* the eggs which extrude polar bodies have undoubtedly received an optimum treatment, whereas the ones that do not form polar bodies have apparently been incompletely activated. But the former have retained only one fourth of the original chromatin of the egg. The ability of the *Urechis* egg to divide would thus appear to depend upon the amount of chromatin present relative to the cytoplasm. However, it is conceivable that by means of other agents or by additional treatment the eggs that extrude two polar bodies may be made to develop, since in the sea-urchin egg fairly normal plutei have been obtained from eggs which contain the haploid number of chromosomes.

Summary

1. The eggs of *Urechis* may be activated by hypotonic solutions ranging from distilled water to 80 per cent sea water, and trochophores indistinguishable from those produced by fertilized eggs may be obtained.

2. The time of exposure necessary to bring about activation increases with increased concentration of sea water.

3. The activated eggs may extrude two, one or no polar bodies. When the exposure is such as to produce 100 per cent activation,

two polar bodies are extruded on practically all the eggs, but to either side of this optimum exposure the proportion of activated eggs with no polar bodies increases. Eggs with one polar body occur in small numbers, and the single polar body is equal in size to the two polar bodies of the normal egg.

4. Only the eggs with no polar bodies divide and form embryos. Those with two polar bodies or with one polar body may sometimes produce uncleaved swimmers.

5. The eggs which extrude no polar bodies show a very poor response in other respects to the treatment. The eggs which extrude two polar bodies respond to the treatment in very much the same manner as if they had been fertilized. The fact that only the former cleave and develop is inconsistent with the view that for development to be obtained the artificially activated egg must behave like the fertilized egg in its initial response to the treatment.

6. The cleavage of the parthenogenetic egg is quite variable both in form and in time of division.

7. The normal embryos are produced in very small numbers. Even when normal-looking eggs in the eight-cell stage are isolated, less than 2 per cent normal development is obtained.

8. Among the abnormal embryos produced by the parthenogenetically activated eggs, a number of larvæ are found that may be classified in a radially symmetrical group. Such embryos may have the form of hollow blastulæ, early gastrulæ, or late gastrulæ, and their development ends without the appearance of bilateral symmetry.

9. The indentation of the unfertilized egg is found to mark the pole, and with respect to that point the polarity of the artificially activated egg is found to remain unchanged.

10. The first cleavage plane is found to pass through the entrance point of the sperm in 71 per cent of the cases. It may be concluded from this that the sperm is instrumental in determining the plane of bilateral symmetry.

11. It is suggested that in the absence of a localized external agent such as the sperm, it is a matter of chance whether the establishment of bilateral symmetry and other processes associated with the orientation of the embryo may be effected. This would account for the low percentage of normal development and the presence of radially symmetrical embryos.

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